III. REMARKS

Claims 1-27 are pending in this application. By this Amendment, the specification and claims 1 and 7-18 are amended, and claims 19-27 are added. The amendments are supported by the specification and the claims as originally filed. The previously filed claims are renumbered 1-18 as directed by the Examiner on page 2 of the Office Action. The amendments to claims 1, 7, and 13 are supported by, for example, Table 2 and claims 8-12 and 14-18 are amended to clarify the dependencies thereof. Claims 19-27 and the amendments to paragraph [0049] of the specification are supported by the originally filed claims and Table 2 of the specification. Meanwhile, the amendments to paragraph [0021] are supported by the originally filed specification and clarify the scope of the presently claimed invention in order to expedite prosecution. No new matter is added.

The specification is objected for the asserted informalities.

Applicants respectfully submit that this objection has been overcome by the above amendments to the specification. In particular, Table 2 is in agreement with the description of Table 2 as provided in paragraph [0049] of the present specification. Table 2 is titled "Baseline and final values of fibrinolytic measures among 3 t-PA genotype groups following moderate exercise training for six months" and discloses the 3 t-PA genotypes as II, ID and DD. Meanwhile, present paragraph [0049] recited that "Table 2 contains data that also shows that I allele carriers respond best in terms of t-PA activity and antigen levels to exercise training."

For at least the above reasons, Applicants respectfully request withdrawal of the objection to the specification.

Claims 1-18 are rejected under 35 U.S.C. § 102(b) as being anticipated by Väisänen et al. (Thromb. Haemost (1999) 82: 1117-1120). This rejection is traversed.

Applicants submit that Väisänen et al. merely discloses the identification of subjects by 4G4G, 4G5G, and 5G5G (Väisänen et al., page 1118, right column, Table 1). Väisänen et al. does not disclose "identifying a subject with ... at least one I allele and/or genotype at the (t-PA) gene locus", much less "identifying a subject with at least one 4G allele and/or genotype at the plasminogen activator inhibitor-1 (PAI-1) gene promoter site, and at least one I allele and/or genotype at the (t-PA) gene locus" or engaging a subject with at least one 4G allele and/or genotype and at least one I allele and/or genotype in exercise training for a period of time sufficient to "increase fibrinolysis", "prevent cardiovascular disease", or "ameliorate cardiovascular disease" in the subject (present claims 1, 7, and 13, respectively).

In order to expedite prosecution, Applicants enclose an article by Jern et al. (Arterioscler. Thromb. Vasc. Biol. (1999) 19: 454-459), demonstrating that those of skill in the art would have known how to "identifying a subject with ... at least one I allele and/or genotype at the (t-PA) gene locus (present claims 19-27). Further, Applicants note the 3 t-PA genotype groups, II, ID, and DD, disclosed by Table 2 in the present specification.

As Väisänen et al. fails to disclose all of the elements of the presently claimed invention, Applicants maintain that those of skill in the art would not have found the

presently claimed invention anticipated by Väisänen et al. Thus, Applicants respectfully request reconsideration and withdrawal of the rejection of claims 1-18 under 35 U.S.C. § 102(b) as being unpatentable over Väisänen et al.

Claims 1-18 are rejected under 35 U.S.C. § 112, first paragraph, for lack of enablement. This rejection is traversed.

Applicants respectfully submit that the specification provides sufficient disclosure to enable present claims 1-18. In particular, Applicants submit that a correlation between the specific genotype of a subject and the response (with regard to fibrinolysis) of that subject to exercise training is disclosed by the present specification. For example, the specification discloses the following:

> [0012] PAI-1 acts to inhibit the fibrinolysis process by inhibiting the actions of t-PA. t-PA is a tissue plasminigen activator and its presence stimulates fibrinolysis. By inhibiting t-PA, PAI-1, therefore, also inhibits fibrinolysis.
>
> [0013] The term "t-PA antigen" is used herein to refer to the t-PA composition that stimulates fibrinolysis.
>
> [0014] The term "t-PA activity" is a description of the rate of chemical and enzymatic reactions caused by the presence of t-PA antigen with and without the presence of PAI-1.

(Specification, paragraphs [0012]-[0014]); and

> [0048] The results set forth in Table 1 show that PAI-1 levels in subjects change with moderate exercise training. Further, the data shows that there is a tendency for subjects with a 4G/4G genotype to respond better than a subject with a 4G/5G or a 5G/5G genotype. The data of Table 1 also shows that t-PA activity also changes in relation with the introduction of moderate exercise. The results describe a tendency for subjects with a 4G/4G genotype to respond better to exercise treatment that individuals with a 4G/5G or 5G/5G genotype. Table 1 also shows that t-PA antigen levels are also adjusted by exercise and that there is a tendency for

> subjects with a 4G/4G genotype to respond better to exercise treatment than a subject with a 4G/5G genotype. Therefore, the data shows that 4G homozygotes respond the best to exercise, with 5G homozygotes either responding less beneficially or not at all.

(Specification, paragraph [0048]) (emphasis added).

As such, the decreased PAI-1, increased t-PA activity, and decreased t-PA antigen reported in the subjects with at least one 4G allele and/or genotype at the PAI-1 gene promoter site after moderate exercise training for six months, increased fibrinolysis in the subjects. Further, Applicants note the increased t-PA activity and decreased t-PA antigen (P ANOVA = 0.054) reported in the subjects with at least one I allele and/or genotype after moderate exercise training for six months, increased fibrinolysis in the subjects (see Table 2).

Improving the levels of fibrinolysis in subjects prevents the development of or alleviates the symptoms of cardiovascular disease, as fibrinolysis is a "process by which a fibrin clot is removed from the site of vascular injury during the healing process" (Specification, paragraph 3). As such, it is clear that removal of a fibrin clot or prevention of fibrin clots at the site of vascular injury will assist in preventing or ameliorating cardiovascular disease in a subject. See also, paragraph [0004] of the present specification.

Applicants note that a reference or control is available in that the baseline of the subjects was measured prior to the exercise period, such that the "change" may be calculated between the final values of the fibrinolytic measures and the baseline values (see, e.g., Table 1 and Table 2).

Applicants also note that the study reported in the 2001 PhD dissertation of Tiyasangthong, which Applicants emphasize is not prior art under 35 U.S.C. § 102, was expanded to a larger population size and reported in Table 1 of the present application (see Table 1). As such, the small population size data within the disclosure on pages 95-96 and Table 7 of the 2001 PhD dissertation of Tiyasangthong, noted by the Examiner on page 9 of the Office Action, was incorporated into the larger population size data in Table 1 of the present specification, as the number of subjects was increased for the PAI-1 genotypes between the filing of the PhD dissertation and the present application. Accordingly, Applicants submit that the data in the 2001 PhD dissertation of Tiyasangthong also does not demonstrate an unpredictability of associating PAI-1 genotype with exercise-induced increases in fibrinolysis.

As sufficient support is provided for the presently claimed invention in the specification and the originally filed claims, Applicants submit that those of skill would have been able to use the presently claimed invention without undue experimentation. For at least the above reasons, Applicants respectfully request reconsideration and withdrawal of the rejection of claims 1-18 under 35 U.S.C. § 112, first paragraph.

In response to the Examiner's request for information under 37 C.F.R. § 1.105, Applicants submit that the PhD dissertation cited by the Examiner (Tiyasangthong, O., "Effects of a common genetic polymorphism and exercise training on fibrinolysis in men and women aged 50-70," PhD Dissertation, University of Maryland, College Park, ISBN 0-493=493-48931-2, pp. 1-172) was made publicly available on January 22, 2003.

Please see the subheading "Date & Time Last Tr 20030122131139.0" (emphasis added) on the enclosed printout of the online catalogue entry for the PhD dissertation.

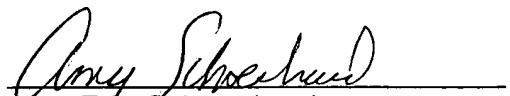
In view of the foregoing, reconsideration of the application, withdrawal of the outstanding rejections and the prompt issuance of a Notice of Allowability are respectfully solicited.

IV. Conclusion

For at least the above reasons, Applicant respectfully submits that this application is in condition for allowance and requests favorable action thereon. If the Examiner believes that anything further is desirable in order to place this application in even better condition for allowance, the Examiner is invited to contact Applicant's undersigned representative at the telephone number listed below to schedule a personal or telephone interview to discuss any remaining issues.

In the event this paper is not considered to be timely filed, Applicant hereby petitions for an appropriate extension of time. The fee for this extension may be charged to our Deposit Account No. 01-2300, referring to Attorney Docket No. **108172-00097**. Please charge any fee deficiency or credit any overpayment to Deposit Account No. 01-2300, referencing Attorney Docket No. **108172-00097**.

Respectfully submitted,



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Enclosures: Petition for Extension of Time (3 months)
Reference (1)
Web Page (2 pages)
Claim Transmittal

Gene Polymorphism of t-PA is Associated With Forearm Vascular Release Rate of t-PA

Christina Jern, Per Ladenvall, Ulrika Wall, Sverker Jern

Abstract—We have observed marked interindividual differences in release rates of tissue-type plasminogen activator (t-PA) among healthy subjects. The objective of the current study was to test the hypothesis that there is an association between a genetic variation at the t-PA locus and the in vivo release rate of t-PA. Fifty-one healthy males were studied at rest in the morning and 27 of these were also subjected to a mental stress test. Net release rates of total t-PA across the forearm vascular bed were calculated as the product of the venoarterial concentration gradient and forearm plasma flow. Zygosity for an Alu-repeat polymorphism in intron 8 of the t-PA gene was determined by a polymerase chain reaction. Basal t-PA release rates differed markedly by genotype (ANOVA, $P<0.05$); subjects homozygous for the insertion had a significantly higher release rate (mean 10.9 ng · min^{-1} · L^{-1}, n=19) than both heterozygotes (4.5 ng · min^{-1} · L^{-1}, n=26) and subjects homozygous for the deletion (0.9 ng · min^{-1} · L^{-1}, n=6). After 2 minutes of mental stress release rates had increased approximately 2-fold in all groups. Arterial and venous plasma levels of t-PA were unrelated to genotype. In conclusion, the current results provide the first evidence of an association between a common genetic variation at the t-PA locus and interindividual differences in net release rates of t-PA in vivo. The relationship is not reflected by circulating steady-state plasma levels and can thus not be disclosed by conventional venous plasma sampling. (*Arterioscler Thromb Vasc Biol.* 1999;19:454-459.)

Key Words: genetics ■ tissue-type plasminogen activator ■ blood flow ■ secretion ■ stress

Genetic factors have been shown to influence plasma protein levels for several hemostatic factors.[1] Common genetic variations (polymorphisms) at the fibrinogen, factor VII, and plasminogen activator inhibitor type 1 (PAI-1) loci have been associated with interindividual differences in the basal steady-state plasma level of the respective proteins.[1-4] In addition, responses to environmental factors have been shown to differ by genotype.[1]

A polymorphism has been identified at the tissue-type plasminogen activator (t-PA) locus on chromosome 8, which consists of the presence or absence of a 311 bp *Alu* sequence in intron 8.[5] The *Alu* insertion probably arose early in human evolution, and a number of populations have been found to be dimorphic for its presence or absence.[6,7] A similar polymorphism in the angiotensin converting enzyme (ACE) gene has been shown to explain approximately 50% of the variability of plasma ACE levels between individuals.[8] In contrast, it was recently reported that t-PA genotypes did not correlate with basal plasma levels of t-PA.[9-11] However, in view of the complex regulation of the steady-state plasma level of t-PA, this observation does not exclude the possibility of an association between genotype and local t-PA release rates. The systemic concentration of t-PA not only is dependent both on secretion and clearance but also on its rate and degree of complex-formation with PAI-1.[12] This is due to the fact that the t-PA/PAI-1 complex is cleared at a slower rate than free t-PA.[13] It follows that an increased plasma concentration of PAI-1 will be paralleled by an increased plasma concentration of total t-PA.[13] Consequently, the systemic plasma level of t-PA is unlikely to directly reflect its secretion rate.

Circulating t-PA is derived from vascular endothelial cells.[14] It is the key enzyme in the initiation of an endogenous fibrinolytic/thrombolytic response.[14] Recent data suggest that the local endothelial release rate, rather than the steady-state plasma concentration, of t-PA determines the thrombolytic potential.[14,15] In 1994, our group has developed a modification of the perfused-forearm model to be able to directly measure rates of t-PA release in vivo.[16-18] This method allows instantaneous determinations of local release rates of t-PA secreted from the endothelium without any interference of liver clearance or being confounded by shifts between complexed or free molecular forms. In addition, studying an intact vascular bed has the advantages of preserved innervation, circulation, blood flow pulsatility, and cell-to-cell interaction. In essence, the model is based on simultaneous arterial and venous sampling from the cubital region and a simultaneous plethysmographic measurement of forearm blood flow (FBF). In a series of studies, we have observed marked interindividual variations in net release rates of t-PA in

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Arterioscler Thromb Vasc Biol. is available at http://www.atvbaha.org

healthy subjects.[17–20] The aim of the current study was to test the hypothesis that part of this difference is related to genetic variations between the subjects.

Methods

In vivo experiments of forearm t-PA release were performed in 51 apparently healthy, nonobese, nonsmoking, male subjects. All subjects were without any medication or history of cardiovascular disease. The investigation was based on earlier studies in our laboratory performed to investigate release mechanisms of t-PA. The nature, purpose, and potential risks of the study were carefully explained to each subject before informed consent to participate was obtained. The protocol was approved by the Ethics Committee of the University of Göteborg, and the study was conducted according to the Declaration of Helsinki. All procedures were performed in accordance with the guidelines of our laboratory. Experiments were performed after an overnight fast (at least 10 hours) and commenced at 8:30 AM.

Catheterization

An arterial polyethylene catheter (Viggo Products, British Viggo) was introduced percutaneously by the Seldinger technique into the brachial artery of the nondominant arm and advanced 10 cm in the proximal direction. An indwelling cannula (Venflon, Viggo) was introduced retrogradely into a deep antecubital vein of the same arm for venous blood sampling from the muscle vascular bed.

Hemodynamic Monitoring

Intraarterial blood pressure was recorded continuously by an electrical transducer (EMT 35, Siemens-Elema) and a Mingograph 82 (Siemens-Elema). Mean arterial pressure was obtained by electrical damping of the pressure signal. The electrocardiogram (ECG) was also continuously monitored on the Mingograph. Venous occlusion plethysmography with a mercury-in-rubber strain-gauge was used to assess FBF.[21] FBF, in $ml \cdot min^{-1} \cdot L^{-1}$ tissue, was calculated from 3 to 5 separate recordings after each blood sampling. Intra- and inter-observer coefficients of variation were, on the average, 5.6% and 4.6%, respectively.

Experimental Design

After the catheterization and application of recording devices, subjects rested 60 minutes in the supine position in a dimly-lit and sound-proof room. Blood samples were then obtained twice 10 minutes apart at rest. Blood sampling procedures were strictly controlled and standardized to avoid sympathoadrenal activation.[16–18] In the subjects exposed to mental stress, additional blood samples were obtained 2 minutes after the start of the stress test.

Mental Stress

A subgroup of 27 subjects performed mental test (mental arithmetic, MA) according to the highly standardized procedure we have previously described in detail.[22,23] The stress test has been shown to induce a reproducible hemodynamic activation.[23]

Blood Sampling

Arterial and venous blood samples were drawn simultaneously by 2 persons from the catheters. The first 3 to 4 mL of blood were always discarded. Blood samples were collected in tubes containing 1:10 0.45 mol/L sodium citrate buffer, pH 4.3 (Stabilyte, Biopool AB). Catheters were flushed with heparinized (5 IU/mL) saline after each blood sampling. The tubes were kept on ice, and plasma was isolated within 60 minutes by centrifugation at 4°C and 2000*g* for 20 minutes. Plasma was immediately frozen and stored in aliquots at −70°C.

Determination of Plasma t-PA Concentration

An enzyme-linked immunosorbent assay (ELISA) was employed for the quantitative determination of total t-PA antigen (TintElize® t-PA, Biopool AB). The assays are based on the double-antibody principle. Free t-PA and t-PA in complex with inhibitors are detected with equal efficiency.[24] The free, active fraction of t-PA (t-PA activity) was determined by a bioimmunoassay (Chromolize® t-PA, Biopool AB). Active t-PA is expressed in μg/L using the specific activity of 600 IU/μg.[20] All samples from 1 individual were assayed in duplicate on the same microtest plate. Intra-assay coefficients of variation were 2.9% and 2.7% for total and active t-PA, respectively.

Hematocrit

Hematocrit was determined in duplicate on arterial blood using a microhematocrit centrifuge (Hettich Hematokrit, Hettich Zentrifugen) with a coefficient of variation of <1%.

Net Release Rate of Total t-PA

Venoarterial concentration gradients (AV-gradients) of each individual were computed by subtraction of the plasma level of total t-PA measured in simultaneously collected venous and arterial blood. A positive difference indicates a net release and a negative net uptake. Individual forearm plasma flow was calculated from FBF and arterial hematocrits corrected for 1% trapped plasma. Individual net release or uptake rates were calculated from the AV-gradient times plasma flow per unit of time and L forearm tissue.[16–18] The following formulas were used:

$$FPF = FBF \times ([101 - \text{hematocrit}]/100)$$

$$\text{Net release} = (C_V - C_A) \times FPF,$$

where C_V denotes venous plasma concentration, and C_A denotes arterial concentration.

Net Increment of Active t-PA

Forearm net increment of active t-PA was calculated with the same formula as net release of total t-PA. However, as regards active t-PA, local net flux may not only reflect tissue release/uptake but also possible shifts between the complex-bound and free fractions of t-PA on passage through the forearm vascular bed.[19,25] To signify this fact, the term net increment is used instead of net release for active t-PA.[19]

Extraction and Amplification of Genomic DNA

Nucleated cells were prepared from frozen EDTA-anticoagulated whole blood according to Sambrook et al.[26] Genomic DNA was extracted by a salting out procedure[27] and amplified by the polymerase chain reaction (PCR) with oligonucleotide primers encompassing the polymorphic region in intron 8 of the PA gene.[5] The forward and reverse primer used were 5′-TCCGTAACAGGACAGCTCA and 5′-ACCGTGGCTTCAGTCATGGA, respectively. The PCR was performed in a final reaction volume of 50 μl containing 0.5 μg of genomic DNA; 20 pmol of each oligonucleotide primer; 100 μM deoxynucleotide triphosphates; 50 mmol/L potassium chloride; 10 mmol/L TRIS-hydrochloric acid (pH 8.3 at 25°C); 1.5 mmol/L magnesium chloride; 0.001% (wt/vol) gelatin; and 1.25 U Taq DNA polymerase (AmpliTaq Gold, Perkin-Elmer/Cetus). The thermocycling procedure (2400 Thermal cycler, Perkin-Elmer/Cetus) consisted of 10 minutes of denaturation at 94°C, followed by 32 cycles of 1 minute at 93°C, 1 minute at 64°C, and 100 seconds at 72°C. The final extension step was prolonged to a total of 7 minutes. A 15 μL sample was analyzed by agarose gel electrophoresis and visualized with ethidium bromide and UV transillumination. Amplification produces a 966-bp fragment from chromosomes with the insertion (*I* allele) and a 655-bp fragment from those without (*D* allele).

To avoid misclassification of *ID* genotypes as *DD*, an independent PCR with a forward primer that recognizes an insertion-specific sequence (5′-GATCACGAGGTCAGGAGAT) was performed on each sample classified as *DD* by the initial PCR. The reverse primer and PCR-conditions were identical as above except for a lower magnesium chloride concentration (1.0 mmol/L) and a slightly higher primer content (30 pmol) and annealing temperature (66°C). The reaction yields a 850 bp amplicon only in the presence of an *I* allele.

Southern Blot and Enhanced Chemiluminescence-Hybridization

To verify specificity of PCR-products, hybridization to a probe directed to a sequence present in fragments from both *I* and *D* alleles

TABLE 1. Clinical and Hemodynamic Characteristics of the Subjects

	Whole Group n=51	DD Group n=6	ID Group n=26	II Group n=19
Age, y	27.2 (1.1)	24.3 (0.7)	27.6 (1.6)	27.6 (1.9)
BMI, kg/m^2	23.7 (0.3)	23.1 (0.9)	23.9 (0.5)	23.7 (0.6)
MAP, mm Hg	83.4 (1.2)	83.5 (3.6)	82.3 (1.5)	84.7 (2.1)
FBF (cath), ml · min^{-1} · L^{-1}	28.3 (1.9)	27.0 (7.3)	28.1 (2.4)	29.0 (3.6)
FBF (non-c), ml · min^{-1} · L^{-1}	26.0 (1.7)	25.1 (5.8)	25.2 (1.8)	27.4 (3.4)
Hct, decimal fraction	0.433 (0.004)	0.439 (0.008)	0.430 (0.005)	0.436 (0.006)

There were no significant difference in any parameter between genotypes (ANOVA $P>0.05$ throughout). BMI indicates body mass index; MAP, mean arterial blood pressure; FBF (cath), forearm blood flow in the catheter arm; FBF (non-c), FBF in the non-catheter arm; and Hct, hematocrit. Numbers are Mean (SEM).

was performed. Amplified DNA from several subjects of each genotype was electrophoresed on an agarose gel and then transferred onto a nylon membrane (Hybond N$^+$, Amersham) using standard Southern blotting methods.[26] Hybridization was performed with 20 pmol biotin-labeled probe (5'-GTCTGGTAGGCACACAGTCT, Scandinavian Gene Synthesis) and 1 unit Streptavidin-POD conjugate (Boerhinger Mannheim). After 3 stringency washes the membrane was transferred to a vessel with detection reagents for enhanced chemiluminescence (RPN 2105, Amersham).

Statistical Analysis

Mean values of the 2 baseline measurements are presented. Standard statistical methods were used. Unless otherwise stated, data are given as mean and SEM. Student's *t*-test was used to test the probability that the AV-gradients or the calculated net release/uptake indices were different from zero. Responses to mental stress were evaluated by 1-way analyses of variance (ANOVA) for repeated measures with subject as random factor. One- and two-way ANOVA was also used to assess whether baseline variables and responses to mental stress differed by genotype. Post-hoc analysis was performed by Fisher's protected least significant difference procedure. The association between 2 variables was evaluated by the correlation coefficient. The statistical analysis was performed after logarithmic transformation of plasma levels of total and active t-PA. Significance tests were considered significant at $P<0.05$ (2-tailed test).

Results

Genotyping

Southern blot hybridization confirmed specificity of the PCR-products. When samples classified as *DD* by the initial PCR were subjected to a second amplification with an insertion-specific primer, 1 sample appeared that would have been mistyped if this additional PCR had not been applied. Frequency of the *I* allele was 0.63 (95% confidence intervals 0.53 to 0.72), which is in accordance with recently published data for other Scandinavian populations.[6] The number of the *II*, *ID*, and *DD* genotypes were 19, 26, and 6, respectively, which was similar to the expected numbers as predicted by the Hardy-Weinberg equilibrium (*II*:*ID*:*DD*=20:24:7).

Association Between Genotype and Net Release Rate of t-PA

The distribution of age, body mass index, or hemodynamic variables did not differ between genotypes (Table 1). Net release rate of total t-PA at rest showed a significant and graded relationship to genotype (Figure 1). Net release rate of t-PA was significantly higher in the *II*-group as compared with both the *ID*- and *DD*-group ($P<0.05$), whereas the difference in net release rate between the *DD*- and *ID*-group did not attain statistical significance. Forearm net increment of active t-PA showed a similar graded relationship with genotype (Table 2). However, the step-up of net increment of active t-PA across genotypes was less marked and fell short of statistical significance. Arterial and venous plasma concentrations as well as the AV-gradient of both total and active t-PA were unrelated to genotype (Table 2).

There was no significant correlation between the net release rate of total t-PA and the arterial plasma level of either total or active t-PA ($r=0.10$ and -0.16, respectively, NS for both). The arterial plasma concentration of total t-PA showed an inverse correlation both to the arterial plasma level and forearm net increment of active t-PA ($r=-0.48$ and -0.46, respectively, $P<0.01$ for both). In contrast, there was a direct correlation between the plasma concentration and net increment of active t-PA ($r=0.45$, $P<0.01$).

In the subgroup exposed to mental stress, a similar relation between genotype and resting as well as stimulated net release rates of total t-PA was observed (Figure 2). After 2 minutes of mental stress both the arterial plasma concentration and forearm net release of total t-PA had increased as compared with baseline ($P<0.05$ throughout). It is of note that the relative increase in t-PA release was of the same magnitude through genotypes. Induction of sympathoadrenal activation was verified by increments in heart rate by on average 15 beats/min and mean arterial blood pressure by 20 mm Hg ($P<0.001$ throughout). Mental stress also induced a slightly enhanced FBF in both arms ($P<0.05$) and an

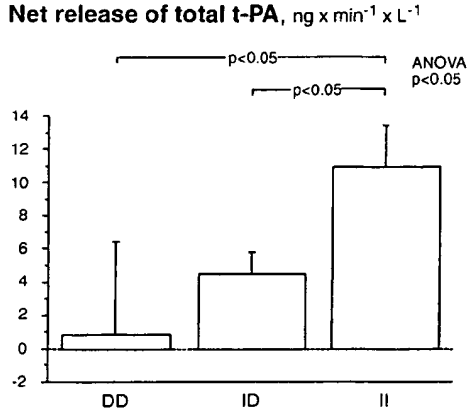

Figure 1. Forearm net release rates of total t-PA at rest in relation to genotype. N=51 (*DD*:*ID*:*II*=6:26:19). Means and SEM error bars.

TABLE 2. Plasma Concentrations, AV-Gradient and Net Release Rate of Total and Active t-PA at Rest

	Whole Group n=51	DD Group n=6	ID Group n=26	II Group n=19	ANOVA
Total t-PA					
Arterial, μg/L	5.84 (0.42)	6.90 (0.88)	5.63 (0.46)	5.81 (0.91)	NS
Venous, μg/L	6.18 (0.47)	7.24 (0.76)	5.89 (0.46)	6.23 (1.08)	NS
AV-gradient, μg/L	0.33 (0.14)*	0.34 (0.41)^{ns}	0.26 (0.08)*	0.43 (0.34)^{ns}	NS
Net release, ng · min^{-1} · L^{-1}	6.5 (1.4)†	0.9 (5.5)^{ns}	4.5 (1.3)*	10.9 (2.5)‡	$P<0.05$
Active t-PA					
Arterial, μg/L	0.72 (0.05)	0.65 (0.13)	0.75 (0.07)	0.73 (0.09)	NS
Venous, μg/L	0.81 (0.06)	0.72 (0.15)	0.82 (0.07)	0.83 (0.12)	NS
AV-gradient, μg/L	0.09 (0.02)†	0.07 (0.04)^{ns}	0.07 (0.02)‡	0.11 (0.05)§	NS
Net increment, ng · min^{-1} · L^{-1}	1.3 (0.2)†	0.8 (0.4)^{ns}	1.1 (0.3)‡	1.7 (0.6)*	NS

Significance levels (*t*-test) for AV-gradients and net release rates versus null hypothesis; ns indicates nonsignificant, $P>0.05$; *$P<0.05$, ‡$P<0.01$, †$P<0.001$, and §$P=0.06$. *P*-values by ANOVA indicates differences by genotype. Numbers are mean (SEM).

increase in arterial and venous plasma concentrations of active t-PA ($P<0.01$ throughout). The were no significant differences in the response pattern to stress between the different genotypes for either hemodynamic or t-PA variables.

Discussion

This is the first study to show an association between a genetic variation at the t-PA locus and interindividual differences in the in vivo release rate of t-PA. The current results show that there is a graded increase in forearm t-PA release rate with the number of *I* alleles. This association is, however, not reflected by steady-state plasma concentrations of t-PA. This is likely to be due to the fact that the steady-state plasma concentration of t-PA not only is dependent on its rate of secretion in combination with the clearance rate but also on the degree of complex formation. That the systemic plasma level of t-PA is a poor marker of t-PA secretion is supported by the current findings of an absence of a correlation between net release rates of total t-PA and plasma levels of either total or active t-PA.

Interpretation of the current finding of a relation between local t-PA release rate and genotype is complicated by the fact that there are 2 pathways of t-PA secretion from endothelial cells: constitutive and regulated secretion.[14,15] In constitutive secretion, the newly synthesized protein continuously leaves the Golgi compartment in transport vesicles to fuse with the cell membrane. By contrast, in regulated secretion, the protein is directed toward an intracellular storage compartment and is only secreted after the cell has been appropriately simulated. An endothelial granule for t-PA has recently been demonstrated,[28] from which regulated secretion is mediated by activation of G-protein coupled cell surface receptors.[14] While cultured unstimulated endothelial cells secrete t-PA by the constitutive pathway,[29] it still remains to be determined whether basal t-PA secretion (and thus steady-state plasma levels) in humans is maintained by constitutive or regulated secretion.[14,15]

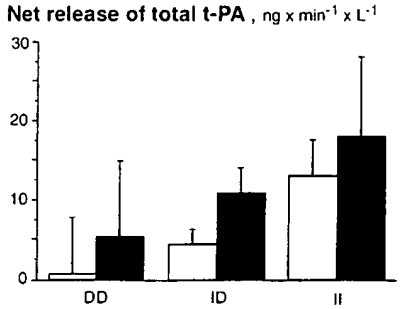

Figure 2. Forearm net release rates of total t-PA in response to acute mental stress. N=27 (*DD*:*ID*:*II*=5:14:8). Open bars represent values at rest and closed bars values after 2 minutes of mental stress. Means and SEM error bars.

The assumption that regulated secretion is the main pathway of t-PA release in vivo, is supported by the finding that, in the intact rat, inhibition of protein synthesis does not result in a reduction of t-PA plasma levels until after several hours[15]. If this also holds true in humans, the current results may indicate that zygosity for the t-PA gene variation determines regulated rather than constitutive release. If so, however, it follows that *DD*-genotypes would completely lack stimulated t-PA release during baseline conditions. This could either indicate that endothelial cells of *DD*-genotypes are insensitive to the stimulatory mechanism that maintain basal regulated release in the other genotypes or have a diminished storage pool of t-PA. However, because *DD* subjects showed a t-PA response to sympathoadrenal stimulation of almost identical magnitude, as did individuals with *ID* or *II* genotypes, it is unlikely that unresponsiveness to stimulation explains the lack of basal release in the *DD*-group.

Instead, the similarity of the stimulated t-PA responses in combination with a marked gradient of basal release rates among the 3 genotypes seems to be more easily reconciled with the interpretation that zygosity is linked primarily to constitutive t-PA secretion. The possibility that constitutive secretion is the main pathway of basal t-PA release in vivo is also supported by the stability of steady-state plasma t-PA levels. Seemingly at variance with this interpretation of the current results, t-PA secretion from cultured umbilical vein endothelial cells has been reported to be unrelated to t-PA genotype.[30] However, endothelial cells in vivo are constantly exposed to stimuli, such as fluid mechanical forces and

humoral factors, known to enhance endothelial t-PA gene expression.[31,32] If the observed relation between genotype and basal net release of t-PA is dependent on such a stimulation, it would not be expected to appear in endothelial cell culture systems.

Whereas the net release of total t-PA was significantly related to genotype, the net forearm increment of active t-PA showed a weaker association with genotype. This is not unexpected in view of our previous findings on local t-PA kinetics. In vitro studies show that t-PA is released in its free, active form.[29] However, newly secreted t-PA will, when it enters the blood stream, very rapidly complex bind to PAI-1 (ie, second order rate constants above 10^7 mol · L^{-1} · s^{-1}). It follows that the net flux of active t-PA across a vascular bed in vivo reflects not only its local release rate but also the kinetics of this local complex-binding to inhibitors.[19,25] This phenomenon is illustrated by our recent observations that the arterial plasma level of PAI-1 is inversely correlated to forearm increment of free, active (but not total) t-PA.[19] In fact, when the inflowing arterial blood contained sufficiently high levels of PAI-1, the abundant amounts of the inhibitor were shown to complex-bind almost all t-PA molecules that were released locally, and the output of free t-PA from the forearm approached zero.[19] In the current study there was an inverse correlation between the plasma level of total t-PA and both the plasma level and net forearm increment of active t-PA. Again, this is most likely explained by local complex-formation, as it is well known that the plasma level of total t-PA show a direct correlation with plasma PAI-1.[33–35] It follows that when assessing local t-PA secretion in vivo, measurements of total t-PA, that is not confounded by local complex-formation, is superior to that of active t-PA.

There are some earlier data to support the notion that the release rate of t-PA in vivo might be genetically determined. Several authors have reported familial clustering of low t-PA secretion as measured by venous occlusion (VO).[36–38] In these studies, relatives to patients with both a history of venous thrombosis and a poor VO-response were investigated. A very modest increment of total t-PA in the brachial vein was observed in a high proportion of both symptomatic and asymptomatic relatives.[38] As the increase in t-PA in response to VO mainly reflects the basal endothelial secretion in the occluded limb,[39] these results are in line with the current findings of an association between genotype and forearm release rates of t-PA at rest.

The mechanism behind the observed association between t-PA genotype and net release rates of t-PA remains to be determined. It seems rather unlikely that an intronic *Alu* insertion polymorphism should have a direct effect on protein production. The most plausible explanation is that the insertion polymorphism is in linkage disequilibrium with functional variations elsewhere at the t-PA gene locus. Thus, the current findings prompt the search for other common polymorphisms at the t-PA locus and to relate such putative variations to expression and release rates of t-PA.

In conclusion, the current results provide the first evidence of an association between a common genetic variation in the t-PA gene and interindividual differences in net release rates of t-PA in vivo. A graded relationship between the number of the *I* alleles and forearm release rate of t-PA was observed. However, the genetic variation is not reflected in circulating plasma t-PA levels, and the altered local t-PA release rate cannot be disclosed by conventional venous plasma sampling. This is in line with the assumption that the level of circulating t-PA is a poor marker of t-PA secretion.

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